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Recombinant poliovirus 3C protease

The enzyme application to protein specific fragmentation

E.P. Sablina and V.K. Antonov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, USSR

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Active 3C protease of poliovirus 1(M) was obtained when cloning and expressing fragment *Hin*dII-*Hin*dIII (bases from 5240 to 6056) of cDNA in vector pTTQ8 in *E.coli* cells. As shown, fragment 3D of polymerase covalently bound to 3C does not deprive the enzyme of its specific proteolytic activity. The absence of 26 N-terminal amino acids in 3C entails its inactivation. The recombinant 3C protease cleaved peptide bond Gln-Gly not only in virus polyprotein, but also in molecules of β-galactosidase and bovine catalase.

Poliovirus; Protease 3C

1. INTRODUCTION

The formation of stable poliovirus proteins includes processing of the single polypeptide-precursor [1,2]. A limited proteolysis is performed by two poliovirus proteases 2A and 3C cleaving peptide bonds Tyr-Gly, and Gln-Gly, respectively [3,4]. Nine of 12 established cleavage sites in the polyprotein of poliovirus are specific to 3C protease [2]. Nowadays a total amino acid sequence of the enzyme (183 amino acids of M_r 19.7 kDa) and a nucleotide sequence of its coding gene have been deciphered [5]. Protease 3C, a cysteine enzyme [6–8] with a unique specificity (cleaves only peptide bond Gln-Gly), is able to split off autocatalytically from the poliovirus polyprotein [8–10]. However, still a lot of problems on 3C function expect their solution. The role of secondary interactions and the spatial structure of the substrate for the enzyme specificity seem rather obscure.

The present paper deals with isolation of the active recombinant 3C protease of poliovirus I(M). The enzyme having a fragment of 3D polymerase in the C-terminus is shown to possess a specific proteolytic activity not only relative to the fragment of a virus polyprotein (cleaves bonds Gln-Gly surrounding peptide VPg at the N- and C-termini), but also cleaves polypeptide bonds Gln-Gly in molecules of β-galactosidase and bovine catalase.

Correspondence address: E.P. Sablina, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya, 16/10 117871 GSP-7, Moscow, V-437, USSR

2. MATERIALS AND METHODS

Constructing of recombinant DNA, preparation of competitive *E. coli* cells and their transformation, clone selection, isolation and analysis of plasmid DNA were performed as described in [11]. Nucleotide sequencing was done according to the Maxam-Gilbert method in a modification of Chuvpilo-Kravchenko [12].

2.1. *Isolation of 'inclusion bodies' from bacterial mass*

Cells HB 101 transformed by vector pTTQ8-PV and cells XL-1 Blue transformed by vector pEKI-PVs were grown in the YT medium [11] with Ap addition (50 μg/ml) up to $D_{600} = 0.2$. After IPTG addition up to the final concentration of 1 mM the cells were grown for 3 h (up to $D_{600} = 1.0$), then collected by centrifugation, washed by cooled buffer A (10 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1 mM PMSF) and cell paste was suspended in buffer A (1/100 of the initial volume of the cell culture) containing 1 mg/ml of lysozyme. After 1 h incubation at 4°C the suspension was sonicated and collected by centrifugation, the pellet was washed by cooled buffer A and stored at −70°C.

2.2. *Isolation and purification of polypeptides P1 and P2.*

The proteins of 'inclusion bodies' isolated from 3 liters of bacterial culture and containing polypeptides P1 and P2 were dissolved in 20 ml buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) containing 8 M urea and loaded onto a column with DEAE Sephacel (2 × 10 cm). Elution was performed by the same buffer. The fractions unbound to an ion-exchanger were combined, dialysed against buffer B and applied to a column with cystatin-Sepharose (2 × 3 cm, 2 mg of cystatin/ml of gel). The proteins bound to cystatin-sepharose were eluted by 0.01 M NaOH solution, pH 12, containing 0.1 M NaCl, dialysed against buffer B, concentrated and stored at −70°C.

2.3. *Isolation and purification of protein β-gal-polio*

The proteins of 'inclusion bodies' isolated from 1 l of bacterial culture and containing recombinant protein β-gal-polio were dissolved in 3 ml buffer B with 8 M urea and applied to the column with CL-sepharose 4B (2 × 70 cm). Elution was performed by the same buffer. The fractions containing protein β-gal-polio were combined, dialysed against buffer B, concentrated and stored at −70°C.

2.4. Assay of specific proteolytic activity of polypeptides including 3C protease:

Two μl of 40% dimethylsulphoxide (DMSO) in buffer B (or 2 μl of 0.1 M iodacetamide in the same buffer containing 40% DMSO) was added to 2 μl (2 μg) of the enzyme in buffer B, then heated for 1 h at 30°C, then 2 μl of 0.1 M DTT was added and the mixture was kept for 10 min at 30°C, 15 μl (15 μg) of the substrate in buffer B was added; then followed by 24-h incubation at 30°C. The reaction mixtures were dried on a concentrator Speed-Vac, dissolved in 20 μl of buffer for loading the samples [13] and after 2 min boiling they were applied to NaDodSO$_4$/PAGE.

Proteins were electrophoretically analysed according to the Laemmli method [13].

Cystatin was isolated as described in [15] and immobilized on epoxy-activated sepharose.

3. RESULTS AND DISCUSSION

Two virus-specific polypeptides binding polyclonal antibodies to the fragment of 3C were obtained when cloning and expressing fragment *Hin*dII-*Hin*dIII (bases from 5240 to 6056 [5]) of cDNA poliovirus 1 (M) containing gene for protease 3C (Fig. 1a) in vector pTTQ8 (Fig. 1b). One of them (PI) was formed at autocatalytic cleavage of bond Gln-Gly, adjoined to N-terminus of 3C in the molecule of the recombinant protein. The other (P2) was a product of translation from alternative methionine encoded by bases 5516–5518 [5] of poliovirus cDNA (Fig. 1a) [10]. Sequence analysis

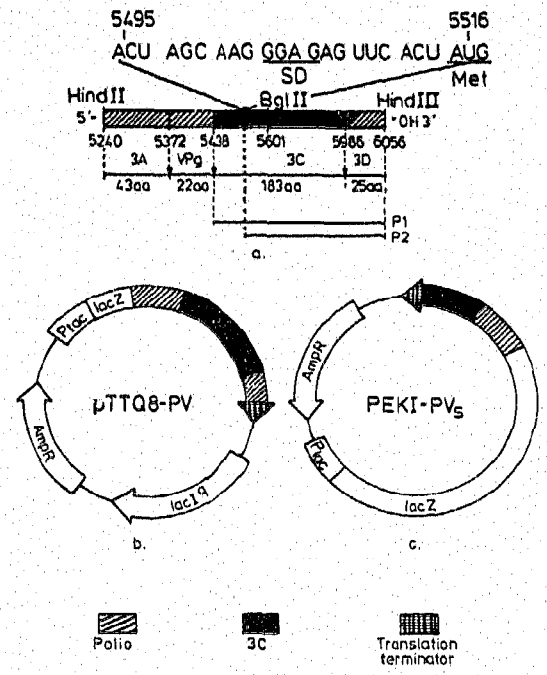

Fig. 1. (a) Fragment *Hin*dII-*Hin*dIII of poliovirus cDNA and the encoded polypeptide containing 43 a/a of 3C protein, 22 a/a of VP_g peptide, 183 a/a of 3C protease and 25 a/a of 3D polymerase. Symbol ↓ designates amino acid pair Gln-Gly recognized by 3C protease of poliomyelitis virus. Bases 5516–5518 of poliovirus cDNA encode methionine initiating translation of an alternative polypeptide. (b) Recombinant vector pTTQ8-PV containing fragment *Hin*dII-*Hin*dIII of poliovirus cDNA. (c) Recombinant vector pEKI-PVs containing fragment *Hin*dII-*Bgl*II of poliovirus cDNA.

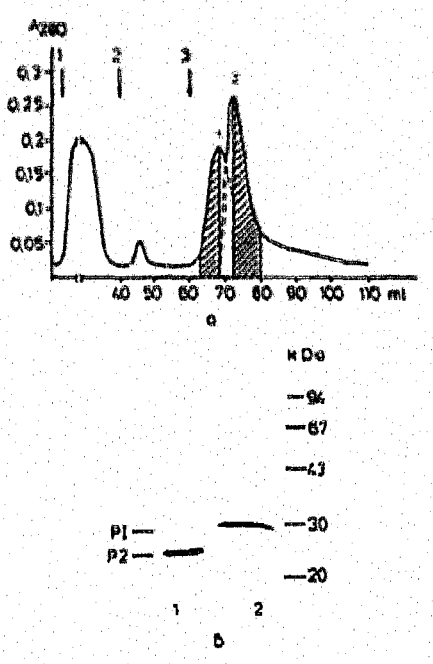

Fig. 2. (a) Chromatography on cystatin-Sepharose of renatured proteins from fractions not bound to DEAE-Sephacel and containing polypeptides P1 and P2. Elution: 1, buffer B; 2, buffer B containing 0.5 M NaCl; 3, 0.01 M NaOH containing 0.1 M NaCl, pH 12.0. (b) Analysis in 12% Na—DodSO$_4$/PAGE protein from peaks I (1) and II (2) marked on chromatograms (a).

showed that the N-terminal sequences of 3C and PI are identical and the sequence of P2 coincides with that of 3C downstream to the residue 27 [15]. Amino acid pair Gln-Gly adjoined to the C-terminus in two cases remained uncleavable, therefore the peptides contained fragment (25a/a) of 3D polymerase besides sequence of amino acids of 3C protease.

Polypeptides P1 and P2 were isolated and purified as described in 'Materials and Methods' (Fig. 2a, b).

The fragment of a virus polyprotein obtained at cloning and expression of fragment *Hin*dIII-*Bgl*II (bases 5240 to 5661 [5], Fig. 1a) of poliovirus cDNA in vector pEKI was used as a natural substrate (Fig. 1c, [16]). The virus polypeptide encoded by fragment *Hin*dII-*Bgl*II and translated in the recombinant protein β-galactosidase-polio did not possess a proteolytic activity

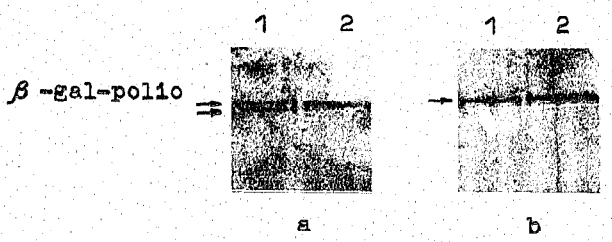

Fig. 3. Assay of specific proteolytic activities of polypeptides P1 and P2. Electrophoregram of β-gal-polio incubated: with P1 (a) and with P2 (b) in the absence (1) and presence (2) of 10 mM iodacetamide.

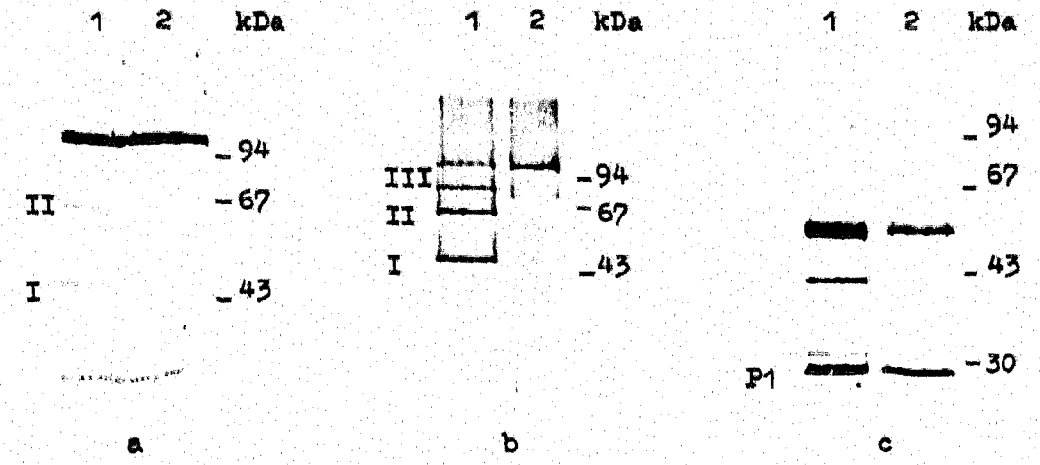

Fig. 4. Analysis of cleavage products of the native (a), denatured (b) β-galactosidase and denatured bovine catalase (c) by poliovirus 3C protease in the absence (1) and presence (2) of 10 mM iodacetamide.

and contained two 3C protease-recognized sites Gln-Gly surrounding peptide VPg at the N- and C-ends (Fig. 1a).

A recombinant protein β-gal-polio purified to homogeneity as described in 'Materials and Methods'.

To define a proteolytic activity the samples of P1 and P2 were incubated with the substrate in the presence or absence of iodacetamide. Electrophoresis showed (Fig. 3a, b) that only polypeptide P1 possessed a proteolytic activity as regards a natural substrate. Enzymatic hydrolysis was suppressed by 10 mM iodacetamide. Polypeptide P2 did not show a proteolytic activity that conforms to an idea about the role of the 3C N-terminal fragment to support an active conformation of the enzyme [17].

Besides the activity of the natural substrate polypeptide P1 displayed a specific proteolytic activity relative to β-galactosidase and bovine catalase and cleaved them at site Gln-Gly.

The amino acid sequence of each of the four identical subunits of *E. coli* β-galactosidase contains 4 sites Gln-Gly in positions 262–263, 563–564, 675–676, and 845–846 [18]. However, after a long incubation of β-galactosidase and the enzyme β-galactosidase was partially hydrolyzed and two fragments formed (Fig. 4a). Deciphering of the N-terminal sequences of the obtained fragments showed that peptide bond Gln-Gly split in position 563–564 that yielded fragments I (50 kDa, N-terminal sequence Gly-Gly-Phe-Val) and II (66 kDa, N-terminal sequence Thr-Met-Ile).

After denaturation of β-galactosidase in buffer with 8 M urea and further dialysis against buffer without urea the rate of the enzymatic hydrolysis increased, here besides fragments I and II fragment III appeared (78 kDa, N-terminal sequence Thr-Met-Ile) (Fig. 4b), that can be explained by cleavage of bond Gln-Gly in position 675–676. An advantageous cleavage of bonds Gln-Gly in positions 563–564 and 675–676 occurs due to their accessibility to the enzyme. This accords well with the fact that the two Gln-Gly sites are located in the proline rich region of the β-galactosidase molecule [18].

Each of four identical chains of the bovine catalase molecule contains 3 sites Gln-Gly in positions 239–240, 351–352 and 397–398. The latter two sites are surrounded by proline residues [19]. The native catalase is not susceptible to the cleavage by 3C but after denaturation with 8 M urea the cleavage of bond Gln-Gly in positions 351–352 and 377–398 yields fragment of M_r 39 kDa (Fig. 4c) and low molecular peptides. The cleavage of bond Gln-Gly in position 239–240 is not apparently observed due to its inaccessibility to the enzyme.

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